Association of Hepatitis C Virus–Specific CD8+ T Cells with Viral Clearance in Acute Hepatitis C

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CD8+ T lymphocytes play a major role in antiviral immune defense. Their significance for acute hepatitis C is unclear. Our aim was to correlate the CD8+ T cell response with the outcome of infection. Eighteen patients with acute hepatitis C and 19 normal donors were studied. Hepatitis C virus (HCV)–specific CD8+ T cells were identified in the enzyme-linked immunospot assay by their interferon-γ (IFN-γ) production after specific stimulation. The highest numbers of IFN-γ–producing HCV-specific CD8+ T cells were found in patients with acute hepatitis C and a self-limited course of disease during the first 6 months after onset of disease, but these numbers dropped thereafter to undetectable levels. The differences in responsiveness between patients with self-limited disease versus patients with a chronic course were statistically significant ($P < .001$). Our data show that the number of IFN-γ–producing HCV-specific CD8+ T cells during the first 6 months after onset of disease is associated with eradication of the HCV infection.

A better knowledge of the role of hepatitis C virus (HCV)–specific CD8+ T lymphocytes is of great importance for the understanding of the pathogenesis of HCV and the development of new therapeutic strategies for chronic HCV infection. In contrast to other viral infections, such as hepatitis B, in which clearance of the virus during the acute phase of disease has been shown to be associated with a strong polyclonal and multispecific cytotoxic T cell response [1, 2], the frequency and significance of HCV-specific CD8+ T cells for viral clearance in acute hepatitis C (aHCV) is unknown. A comparison of the virus-specific CD8+ T cell response in patients with acute self-limited disease versus that in patients with chronic hepatitis C infection may lead to a better understanding of the role of CD8+ T lymphocytes in viral elimination during hepatitis C infection.

Recently, several new methods for determining a specific CD8+ T lymphocyte response have been developed and have yielded new insights into the function and kinetics of a virus-specific CD8+ T cell response [3–5]. Two of the methods measure the interferon-γ (IFN-γ) production of CD8+ T cells after stimulation with viral peptide. The CD8+ T cell numbers are determined either by measuring secreted IFN-γ with the enzyme-linked immunospot (ELISPOT) assay or by cytoplasmic staining for IFN-γ and subsequent fluorescence-activated cell-scanning analysis. The third available measurement method enumerates antigen-specific cytotoxic T lymphocytes (CTLs) by direct staining of virus-specific CD8+ T cells by use of tetrameric complexes of major histocompatibility complex class I glycoprotein plus viral peptide. The data published for human immunodeficiency virus (HIV) and lymphocytic choriomeningitis virus (LCMV) showed that the numbers of identified CD8+ T lymphocytes are similar with the 3 methods and are higher than the numbers derived from limiting dilution analysis and ^{51}chromium (^{51}Cr)–release assay. Among other explanations, this difference may be attributable to the fact that ^{51}Cr-release assays require 1 or several steps of in vitro restimulation, which preferentially lead to expansion of resting memory CTLs, whereas the ELISPOT assay allows detection of finally differentiated effector CTLs, which means that this assay may more accurately reflect the in vivo situation [3–7]. We have chosen to apply the ELISPOT assay, which has been suggested as a candidate for the gold standard in terms of its ability to quantify CD8+ T cells [8], in order to study HCV-specific CD8+ T cells.

The present study addresses the question of whether there is a correlation between the occurrence of HCV-specific CD8+ T lymphocytes and the outcome of infection. To identify the IFN-γ response of virus-specific CD8+ T lymphocytes, a panel of previously described HCV-specific epitopes [9–17] was used for

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Table 1. Patient characteristics.

Patient	Sex	Age, years	HLA class I	Outcome	Peak, ALT U/L	ALT U/L at date of assay	Time between aHCV and assay, months	Therapy
1	M	41	A2, B7	Self-limited	1174	18, 13, 15	6, 9, 13	No
2	F	57	B7	Self-limited	581	12, 10	11, 25	No
3	F	37	B7	Self-limited	828	11	10	No
4	F	42	B8	Self-limited	1728	8, 6, 6	4, 9, 20	No
5	M	47	B7	Self-limited	792	9	4	No
6	M	42	A2, B8	Self-limited	1466	12	6	No
7	F	17	A2	Self-limited	1140	10, 10	2, 10	No
8	M	35	A2, B7	Self-limited	659	659, 15	2, 8	No
9	M	30	A2	Self-limited	1546	1546, 15	2, 7	No
10	F	35	B8	Pending	2232	152, 32, 77	1, 2, 3	No
11	F	34	A2, B7	Relapsed	958	958	1	No
12	M	39	A2, B7	Relapsed	987	62, 42	5, 8	No
13	M	64	A2	Relapsed	949	29, 385	2, 7	No
14	F	64	A2	Relapsed	1730	147, 509	1, 7	No
15	F	55	A2	Chronic	850	850, 24	1, 8	Yes
16	F	75	A2	Chronic	1514	1514	1	No
17	M	21	A2, B7	Chronic	580	431, 18	1, 8	Yes
18	M	57	A2	Chronic	710	141, 184	1, 7	No

NOTE. aHCV, acute hepatitis C virus; ALT, alanine aminotransferase; F, female; M, male.

stimulation of peripheral blood mononuclear cells (PBMC) in 18 patients with aHCV infection, and the IFN-γ production of $CD8^+$ T lymphocytes was measured in the ELISPOT assay. As controls, 19 normal donors were tested according to the protocols used to test the patients. Our results show that viral clearance and resolution of disease in aHCV infection is associated with the occurrence of HCV-specific $CD8^+$ T cells during the first 6 months after onset of disease. The ELISPOT assay proved to be a powerful tool for identifying and enumerating virus-specific $CD8^+$ T cells.

Materials and Methods

Patients. Thirty-seven HLA class I–typed individuals were studied (9 patients with aHCV and a self-limited course, 4 patients with aHCV who became temporarily negative for HCV RNA, 4 patients with aHCV and a primarily chronic course of disease, 1 patient with aHCV and a follow-up within less than 6 months [table 1], and 19 normal donors). The diagnosis of aHCV was based on the following criteria: (1) elevated serum alanine aminotransferase levels—levels that were ⩾20 times above the upper limit of normal; (2) seroconversion of anti-HCV–negative to anti-HCV–positive antibody status by second- or third-generation enzyme-linked immunosorbent assay or recombinant immunoblot assay II; (3) positive polymerase chain reaction (PCR) for HCV RNA in at least the first serum sample (by use of nested PCR [detection limit, 200 eq/mL]); and (4) history of sudden onset of liver disease in previously healthy persons. Other possible causes of acute hepatitis, such as other viral hepatitis, autoimmune hepatitis, alcoholic liver disease, or toxins, have been excluded.

The following 3 groups of patients and a control group were included in the study: (1) patients with aHCV who eliminated the virus permanently and who had a self-limited course of disease; (2) patients with aHCV who became transiently negative for HCV RNA during the first 6 months of disease but who were positive for HCV RNA after 6 months follow-up and who developed chronic infection; (3) patients with aHCV and a chronic course of disease; and (4) normal donors. All normal donors were HCV seronegative.

Isolation of PBMC and proliferation assay. Human PBMC were isolated from heparinized blood by Ficoll-paque density-gradient centrifugation. Briefly, PBMC were washed 4 times in phosphate-buffered saline (PBS) and were resuspended in tissue-cultured medium (RPMI 1640 medium; Gibco, Grand Island, NY) containing 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 10% human AB serum.

PBMC were incubated at 5×10^4/well in 96 U-bottom plates (Costar, Cambridge, MA) for 5 days in the presence of HCV proteins (1 μg/mL) in 150 μL tissue culture medium. On day 5, cultures were labeled by incubation for 16 h with 2 μCi ^{3}H-thymidine (specific activity, 80 μCi/m*M;* Amersham, Buckinghamshire, United Kingdom). Cells were then collected and washed on filters (Dunn, Asbach, Germany) by use of a cell harvester (Skatron, Sterling, VA), and the amount of radiolabel incorporated into DNA was estimated by use of a beta counter (LKB/Pharmacia, Uppsala, Sweden). Triplicate cultures were routinely assayed, and the results are expressed as mean counts per minute (cpm). The stimulation index (SI) was calculated as the ratio between cpm obtained in the presence of antigen and that obtained without antigen. An SI ⩾3 was considered significant.

HCV proteins. Recombinant proteins for antigenic stimulation of $CD4^+$ T lymphocytes were kindly provided by M. Houghton (Chiron, Emeryville, CA), and these proteins comprised the antigen c200 (nonstructural protein NS3 + NS4). All antigens were expressed as COOH-terminal fusion proteins with human superoxide dismutase (SOD) in yeast. Yeast and SOD were tested as controls in each proliferation assay for unspecific stimulation. The purity of the antigens ranged from 80% to 90%.

Synthetic peptides. A panel of peptides that represents 19 previously identified HLA A2-, B7-, and B8-restricted HCV CTL epi-

Table 2. Epitopes (cutoff for a positive response was assessed in normal donors and is indicated as no. of specific spots).

Protein	Amino acid position	Amino acid sequence	Strain or type	HLA class I	Peptide no.	Positive cutoff (specific spots)	Reference
Core	35–44	YLLPRRGPRL	HCV-1	A2	1	11	[9, 10]
	131–140	ADLMGYIPLV	HCV-1	A2	2	10	[10]
E1	220–227	ILHTPGCV	HCV-H	A2	3	9	[15]
	257–266	QLRRIDLLV	HCV-1	A2	4	7	[16]
	363–371	SMVGNWAKV	HCV-H	A2	5	10	[15]
E2	401–411	SLLAPGAKQNV	HCV-1	A2	6	8	[15]
NS3	1073–1081	CINGVCWTV	HCV-1	A2	7	12	[10]
	1169–1177	LLCPAGHAV	HCV-1	A2	8	14	[10]
	1287–1296	TGAPVTYSTY	HCV-BK	A2	9	20	[14]
	1406–1415	KLVALGINAV	HCV-1	A2	10	7	[10]
NS4	1789–1797	SLMAFTAAV	HCV-1	A2	11	4	[10]
	1807–1816	LLFNILGGWV	HCV-1	A2	12	5	[9, 10]
NS4B	1851–1859	ILAGYGAGV	HCV-1	A2	13	13	[9]
NS5	2252–2260	ILDSFDPLV	HCV-1	A2	14	5	[10]
NS5B	2578–2587	RLIVFPDLGV	HCV-1	A2	15	6	[9]
	2727–2735	GLQDCTMLV	HCV-1	A2	16	11	[9]
Core	41–49	GPRLGFRAT	HCV-1	B7	17	12	[12]
	111–119	DPRRRSRNL	HCV-1	B7	18	8	[17]
NS3	1395–1403	HSKKKCDEL	HCV-1	B8	19	10	[13]

NOTE. HCV, hepatitis C virus; NS, nonstructural protein.

topes [9–17] were selected for this study (table 2). All peptides were synthesized with a free amine NH2-terminus and a free acid COOH-terminus by Chiron Mimotopes (Clayton, Australia). Lyophilized peptides were reconstituted at 20 mg/mL in dimethyl sulfoxide (Mallinckrodt, Paris, KY) and were diluted to 1 mg/mL with RPMI 1640 medium (Gibco).

HLA typing. HLA class I typing of subjects was performed on fresh PBMC by the Immunogenetics Laboratory (University of Munich) using standard serologic techniques.

ELISPOT assay. Nitrocellulose-bottom 96-well millititer HA plates (Millipore, Bedford, MA) were coated with 100 μL of the IFN-γ monoclonal antibody (anti–IFN-γ; Mabtech AB, S-10405, Stockholm, Sweden), at a concentration of 15 μg/mL, in PBS and were incubated overnight at 4°C. Unbound antibodies were removed via 4 successive washings with PBS containing 0.05 Tween. The coated wells were filled in duplicate with 100 μL of RPMI medium containing 2×10^5 freshly isolated PBMC, together with the respective HCV peptide (20 μg/mL) or phytohemagglutinin (PHA) (5 μg/mL), and samples were incubated (undisturbed) for 48 h at 37°C in a humidified atmosphere with 5% CO_2.

After incubation, the cells were removed by washing the plates 4 times with PBS. One hundred microliters of the biotin-conjugated anti–IFN-γ (Mabtech AB) was added to each well at a dilution of 1 μg/mL and incubated for 4 h at room temperature. The plates were rinsed 4 times by immersion in PBS and were exposed to 100 μL of streptavidin–alkaline phosphatase (Mabtech AB) for 1 h. Unbound conjugate was removed by washing thoroughly with PBS, and, finally, 100 μL of 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrate solution (Bio-Rad Laboratories, Richmond, CA) was added, and the sample was incubated for 2 h. The color reaction was stopped by extensive washings under running water, and after drying, the number of spots was scored by use of a dissection microscope. Stimulation with PHA was used as a positive control.

Statistical analysis. Analysis of differences among the 3 patient groups and between groups A and B was accomplished by use of the χ^2 test, the Fisher exact test, and the Wilcoxon signed rank test, respectively. For statistical analysis, the results are calculated as specific spots (number of spots in the presence of HCV peptide minus the number of spots without peptide) as follows: nonspecific spots, all individuals tested (median, 1; range, 0–11); patients with acute self-limited hepatitis C (median, 1; range, 0–10); patients with aHCV who became temporarily negative for HCV RNA (median, 1; range, 0–5); and patients with aHCV and a primary chronic course of disease (median, 1; range, 0–11). No statistical difference related to the number of nonspecific spots was found among the different groups (Kruskal-Wallis test).

In order to exclude artifacts, only tests within the 95% confidence interval for negative and positive controls were considered for evaluation. Specific spots greater than the mean control (+2 SD) for the peptides in normal donors were required to be considered positive or significant. The cutoffs for each peptide are indicated in table 2.

Characterization of effectors by specific cell-depletion studies. $CD4^+$ and $CD8^+$ T cells were depleted according to the manufacturer's instructions. Briefly, 10^7cells were depleted by incubation with 20 μL of anti-CD4 or anti-CD8 monoclonal antibody conjugated to ferrous beads (MACS MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany) in 80 μL of buffer (PBS containing 2 m*M* EDTA and 0.5% bovine serum albumin) at 4°C for 30 min. After incubation, the cells were washed, the supernatant was removed, and the cell pellet was resuspended in 500 μL buffer. The conjugate-coated cells were removed by passing through a positive selection column type LS+/VS+ placed in the magnetic field of a magnet (MidiMACS, Miltenyi Biotec). Cells eluted from the bottom of the column were treated as $CD8^-$ and $CD4^-$ fractions, respectively. The beads used here reliably deplete >99% of the target cell population. Purity of the cell fractions was confirmed by flow cytometric analysis (data not shown).

Characterization of effectors by anti-CD8–blocking assay. To

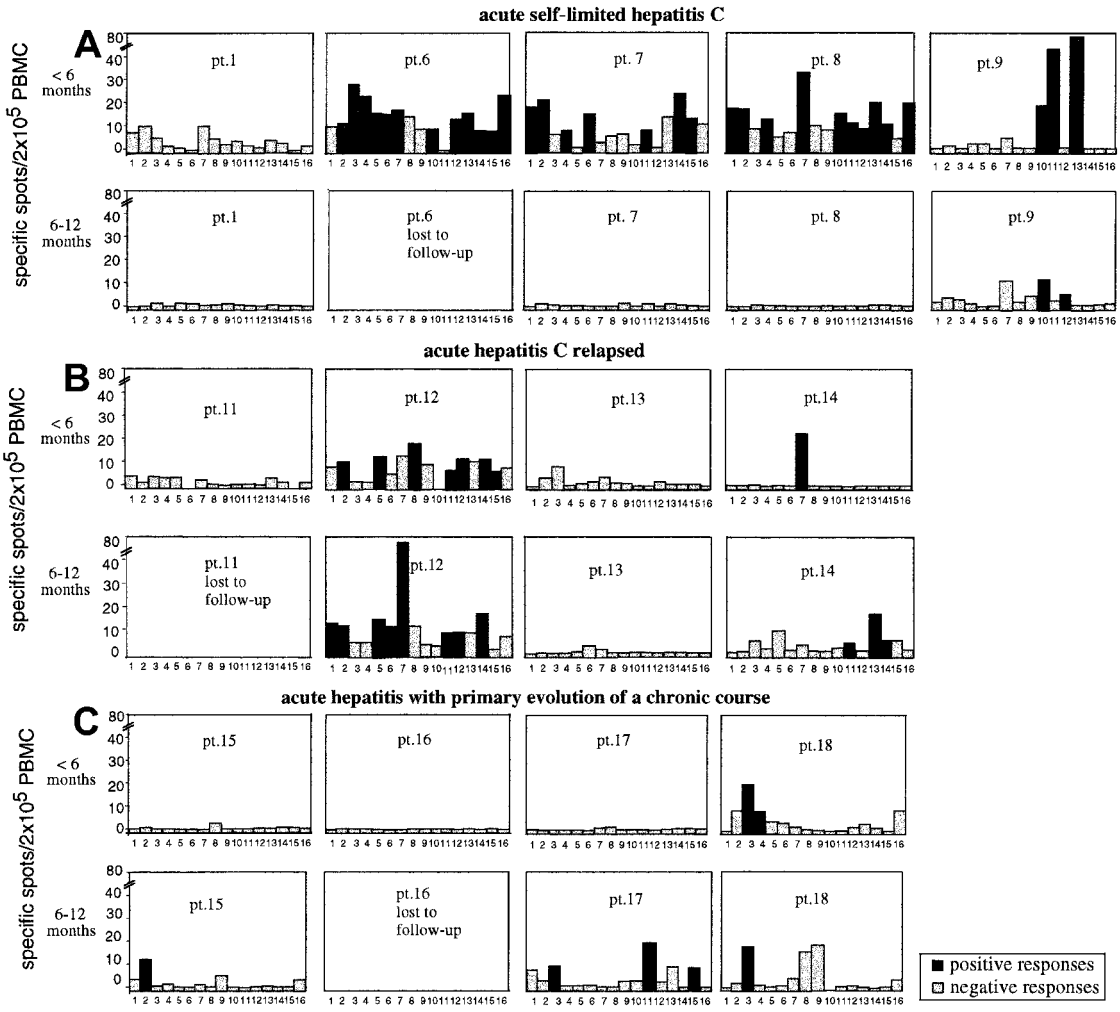

Figure 1. *A–C,* Enzyme-linked immunospot (ELISPOT) results for patients with HLA A2. Peripheral blood mononuclear cells (PBMC) from 13 patients were stimulated with 20 μg/mL of HLA A2–restricted peptides for 48 h, and the interferon-γ production was measured in the ELISPOT assay as described in Materials and Methods. Each bar represents the specific spots/2×10^5 PBMC. The numbers on the abscissa indicate the peptides used (table 2). Specific spots greater than the mean control (+2 SD) for the peptides in normal donors were required before the results were considered positive. Two time points (<6 months and ⩾6 months) for each patient are shown.

further characterize the cell population stained in the ELISPOT assay, the contribution of $CD8^+$ T cells to the IFN-γ production in the ELISPOT assay was determined by incubating PBMC with anti-CD8 antibody (Coulter Immunotech, Marseille, France) at 4°C for 60 min before addition to the ELISPOT assay. After incubation, the cells were washed and resuspended in culture medium.

Results

ELISPOT results for patients with aHCV and a self-limited course. The highest numbers of positive responses were seen during the first 6 months after onset of disease in patients with aHCV and a self-limited course. Four of 5 patients with HLA A2 showed peripheral $CD8^+$ T cells with effector function within 48 h of T-cell–receptor occupancy with >1 HCV peptide, representing a multispecific and significant response during the first 6 months after onset of disease. This responsiveness was directed against structural, as well as nonstructural, epitopes. A follow-up testing between 6 and 12 months after onset of disease was possible in 4 of these patients. The responsiveness seen in the early phase is no longer detectable in 3 of 4 patients or has dropped to borderline positivity in 1 of them (figure 1*A*). Patient 6 was lost to follow-up.

Five patients with acute self-limited hepatitis C and HLA B7 could be tested (figure 2*A*). Positive responses were seen during the first 6 months after onset of disease in patients 1 and 8. For patients 2 and 3, the response was seen between 6 and 12 months after onset of disease. During the first 6 months after

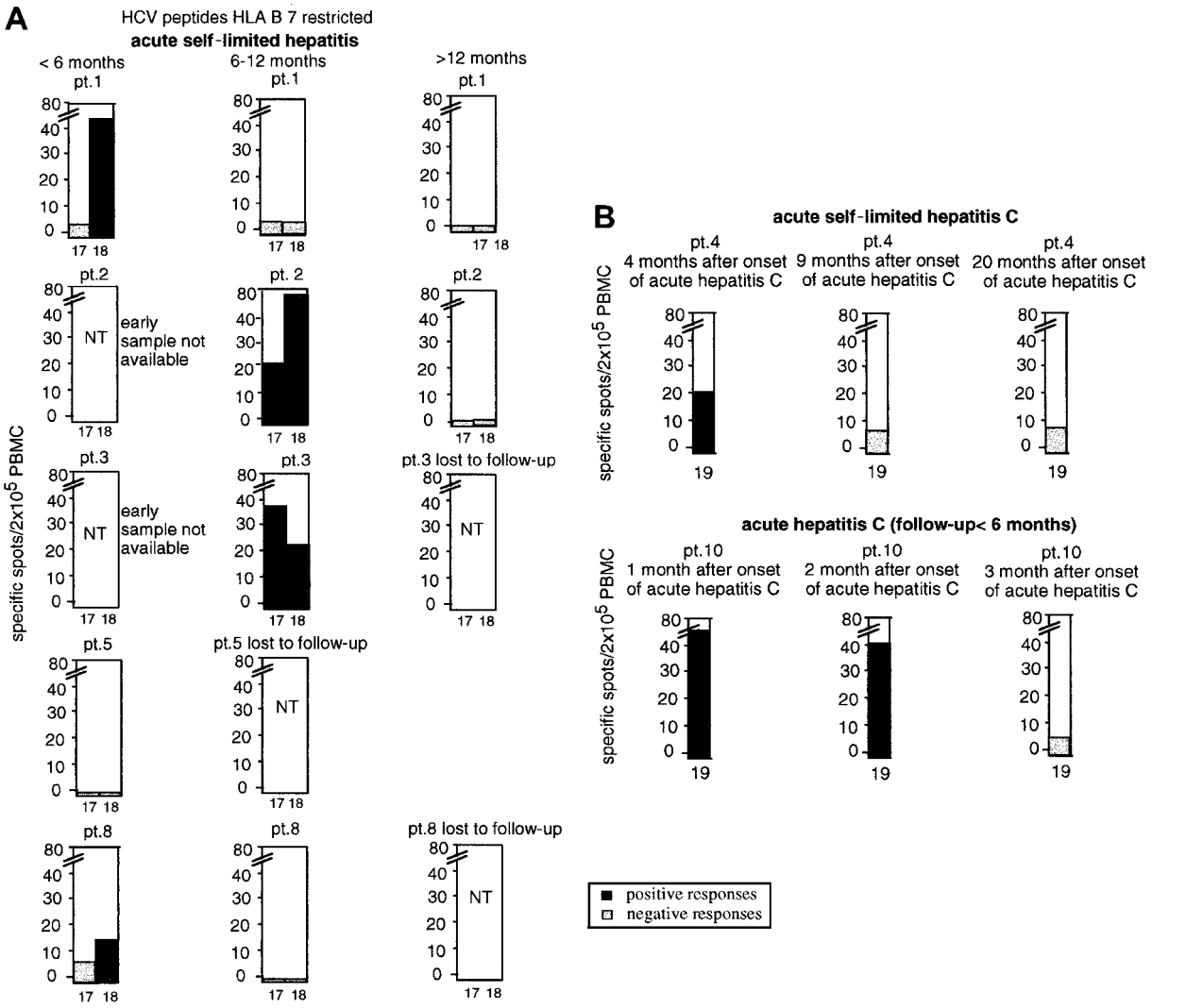

Figure 2. *A* and *B,* Enzyme-linked immunospot (ELISPOT) results for patients with HLA B7 and B8. Peripheral blood mononuclear cells (PBMC) from 7 patients were stimulated with 20 μg/mL of HLA B7–restricted (3 time points are shown for each patient) or HLA B8–restricted (3 time points are shown for each patient) peptides for 48 h, and the interferon-γ production was measured in the ELISPOT assay as described in Materials and Methods. Each bar represents the specific spots/2×10^5 PBMC. Nos. on the abscissa indicate the peptides used (table 2). Specific spots greater than the mean control (+2 SDs) for the peptides in normal donors were required before the results were considered positive. NT, not tested.

onset of disease, no PBMC were available for these 2 patients. Patients 1 and 2 were tested >1 year after onset, and no significant response was detectable at that point. Patient 3 was lost to follow-up (figure 2*A*).

For patients with HLA B8 (total of 2 patients), 1 patient with self-limited disease and 1 patient with a follow-up of 3 months (outcome to be determined) were tested (figure 2*B*). Patient 4 had a significant response to the HLA B8 epitope NS3 1395-1403 during the first 6 months. More than 6 months after onset of disease, this response dropped below the level of significance. For patient 10 (during the first 6 months after onset of disease) a significant response was also found, and this response declined over the course of time to a level that was below the level of significance (figure 2*B*).

ELISPOT results for patients with aHCV and a chronic course. During the first 6 months after onset of disease, 1 of 4 patients with HLA A2 had a bispecific response (patient 18). Between 6 and 12 months after onset of disease, 2 patients who had no response during the first 6 months after onset of disease had a monospecific or multispecific response under specific treatment (patients 15 and 17). Patient 15 started with IFN and ribavirin 6 months after onset of disease, and patient 17 started with IFN monotherapy 3 months after onset of disease. Patient 18, who had a bispecific response at the beginning, displayed

Table 3. No. of positive responses (in relation to total tests performed) for HLA A2–restricted epitopes for patients with acute hepatitis C and self-limited, relapsed, or chronic course of disease during the first 6 months after onset of disease.

Epitopes	HLA restriction	Self-limited	Relapsed	Chronic	Peptide no.
Core 35–44	A2	2/5	0/4	0/4	1
Core 131–140	A2	3/5	1/4	0/4	2
E1 220–227	A2	1/5	0/4	1/4	3
E1 257–266	A2	3/5	0/4	1/4	4
E1 363–371	A2	1/5	1/4	0/4	5
E2 401–411	A2	2/5	0/4	0/4	6
NS3 1073–1081	A2	2/5	1/4	0/4	7
NS3 1169–1177	A2	0/5	1/4	0/4	8
NS3 1287–1296	A2	0/5	0/4	0/4	9
NS3 1406–1415	A2	3/5	0/4	0/4	10
NS4 1789–1797	A2	3/5	1/4	0/4	11
NS4 1807–1816	A2	2/5	1/4	0/4	12
NS4B 1851–1859	A2	3/5	0/4	0/4	13
NS5 2252–2260	A2	3/5	1/4	0/4	14
NS5B 2578–2587	A2	2/5	1/4	0/4	15
NS5B 2727–2735	A2	2/5	0/4	0/4	16

NOTE. NS, nonstructural protein.

a monospecific response 7 months after onset of disease (figure 1*C*). Patient 16 was lost to follow-up.

Since the timing of the assay might have been crucial, assays were performed, if possible, at several time points. However, in our experience and because of the special characteristics of this patient group, material from patients with aHCV could not be supplied on a regular basis, and patients with self-limited disease in particular are not always involved in a close follow-up practice. Therefore, we tested different time points in the patient groups. We decided to present the first available assay because it offers a clear presentation and comparability. For patients 15–17, assays at 2, 3, and 4 months were also performed, and these assays were always negative. Thus, the differences among the patient groups cannot be explained by different timing of assays.

ELISPOT results for patients with aHCV and transiently negative HCV RNA. Patients with aHCV, in whom HCV RNA was transiently undetectable and in whom transaminases were normalized during the first 6 months but who subsequently relapsed, with positive RNA in serum and elevated transaminases, were considered as a separate group. All of these patients relapsed between 3 and 4 months after onset of aHCV. During the first 6 months after onset of disease, 1 of 4 patients had a multispecific response, and 1 patient had a monospecific response. Between 6 and 12 months after onset of disease, patient 12 still had a multispecific response, and patient 14 had a borderline multispecific response. For patient 13, no responsiveness could be detected. Patient 11 was lost to follow-up. Patient 14 was studied in the negative phase of HCV RNA. Patients 11, 12, and 13 were studied in the HCV-RNA–positive phase (figure 1*B*).

ELISPOT results for normal donors. Either zero cells or <10 HCV-specific IFN-γ–secreting cells/2×10^5 PBMC were observed in the presence of HLA-matched HCV-specific peptides in 17 of 19 normal donors. In 1 of the normal donors we detected >10 specific cells/2×10^5 PBMC (representing a positive response) to 5 of the HCV-specific epitopes (range, 11–22 specific spots). These epitopes were located in NS3, NS4B, and NS5B. In 1 normal donor, we observed 30 specific spots to peptide E1 363-371 but no specific spots to the remaining 15 HLA A2 peptides tested in this person.

Previous studies demonstrated that because each spot in the ELISPOT assay represents the cytokine molecules secreted by a single cell, an enumeration of the frequency of effector $CD8^+$ T cells is possible. The highest frequencies of $CD8^+$ T cells specific for 1 HCV peptide in our patients with self-limited disease reached up to 0.2% of all peripheral $CD8^+$ T cells. Since the responses had a multispecific pattern, the percentage of peripheral $CD8^+$ T cells specific for all of the HCV peptides is supposed to be much higher.

In summary, during the first 6 months, the differences in responsiveness for patients with HLA A2 and self-limited disease versus patients with a chronic course of disease ($P < .001$) or versus patients with transiently negative HCV RNA ($P < .001$) were statistically significant. The difference between patients with a primary chronic course of disease versus patients with transiently negative HCV RNA ($P = .052$) failed to prove significance (table 3).

Characterization of effectors. The IFN-γ–secreting cells could be blocked by anti-CD8 antibody, consistent with the expectation that the production of IFN-γ was mediated by $CD8^+$ T cells (data not shown). Moreover, depletion of $CD8^+$ T cells from fresh PBMC completely abrogated the HCV-specific responses. Conversely, depletion of $CD4^+$ cells did not diminish the number of IFN-γ–secreting cells (figure 3).

Correlation of the $CD4^+$/$CD8^+$ T cell response. Addition-

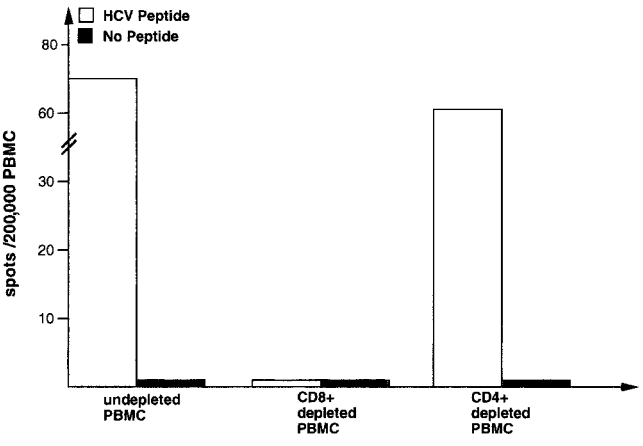

Figure 3. Interferon-γ production on stimulation with HLA class I–restricted hepatitis C virus (HCV) epitope and without stimulation in a 48-h enzyme-linked immunospot assay before and after depletion of $CD4^+$ or $CD8^+$ T cells. Each bar represents the spots/2×10^5 cells, predepletion.

ally, the $CD4^+$ T cell response to recombinant NS3 and NS4 protein was assessed at identical time points in parallel to the $CD8^+$ T cell response in certain cases. Interestingly, except for 1 assay, all patients with a significant $CD8^+$ T cell response also showed a significant $CD4^+$ T cell response at the same time (figure 4). As described recently by Gerlach et al. [18], the $CD4^+$ T cell response was maintained only in patients with a self-limited course of disease (data not shown). The $CD8^+$ T cell response waned to undetectable levels in these patients (figures 1 and 2).

Discussion

The current study was performed to analyze a potential association between the HCV-specific $CD8^+$ T cell response and the course of aHCV infection. As a main result, we found a significant difference during the first 6 months after onset of disease, in terms of responsiveness to a set of HLA-matched HCV-specific CTL peptides, between patients with aHCV and a self-limited course and those with a chronic course of disease.

During the first 6 months after onset of disease in patients with aHCV, a significant $CD8^+$ T cell response directed against multiple class I–restricted HCV epitopes of different regions of the HCV polyprotein correlated with termination of the disease. Four of 5 patients with HLA A2 and a self-limited course of aHCV had positive multispecific responses (during the first 6 months after onset of disease) against structural, as well as nonstructural, HCV epitopes. The significant responsiveness seen during the first 6 months after onset of disease in patients with acute self-limited disease was restricted to this phase and thereafter dropped to undetectable levels. A similar pattern in responsiveness was found in patients with HLA B7 and B8. After viral clearance, no significant difference between the different patient groups was detectable. Calculating the frequency of peripheral HCV-specific $CD8^+$ T cells, percentages of up to 0.2% of the $CD8^+$ T cells were found to be specific for 1 HCV peptide. Recent data for patients with chronic HCV infection have revealed that the frequencies of intrahepatic HCV-specific $CD8^+$ T cells are up to 190-fold higher than the peripheral frequencies [19]. Since liver biopsies are contraindicated in patients with aHCV, studies of the HCV-specific $CD8^+$ T cell response have to rely on blood samples that contain far fewer HCV-specific $CD8^+$ T cells. However, by extrapolating the frequencies found in our patients, one can speculate that the frequencies of $CD8^+$ T cells specific for 1 peptide in the liver of patients with aHCV and a self-limited course could be in the range of 2% of all $CD8^+$ T cells, assuming that the intrahepatic frequencies are 10-fold higher than in the peripheral blood. Furthermore, taking into consideration that the responses are multispecific (e.g., patient 6 with 10 epitopes recognized in the peripheral blood), it is possible that the majority of intrahepatic $CD8^+$ T cells are HCV-specific $CD8^+$ T cells and not unspecific bystander cells. Most likely these numbers still underestimate

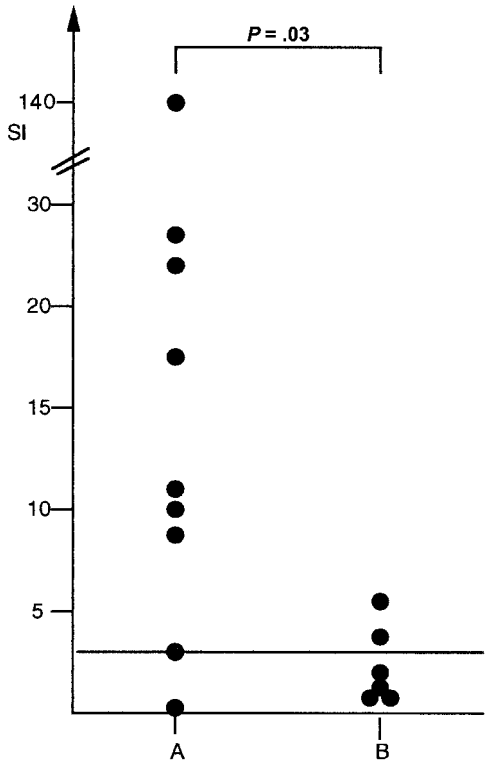

Figure 4. Proliferative $CD4^+$ T cell response for patients with (group A) and without (group B) a significant $CD8^+$ T cell response during the first 6 months after onset of acute hepatitis C virus to recombinant HCV proteins NS3 and NS4 (C-200 Chiron). Results are shown as stimulation index (SI) = ^{3}H-thymidine incorporation of antigen-stimulated peripheral blood mononuclear cells (count per minute [cpm])/unstimulated control (cpm). Values ≥ 3 are considered significant.

the total numbers of HCV-specific $CD8^+$ T cells in the peripheral blood, as well as in the liver, for several reasons. First, the peptides used for stimulation cover only part of the HCV genome and of the potential viral epitopes capable of inducing a $CD8^+$ T cell response. There are certainly $CD8^+$ T lymphocyte specificities that are different from the known ones that are relevant during infection. Second, viral epitopes used for stimulation covered (with their binding motif) only some HLA types, although a cross-reactivity has been shown for several of the presented epitopes [20]. Third, nonreactivity related to sequence difference in the peptides used for stimulation and in the epitopes of the individual patients due to mutations or different HCV strains cannot be ruled out.

In patients with aHCV and a chronic course of disease, only 1 bispecific response was found during the first 6 months after onset of disease. Interestingly, 1 patient mounted a monospecific response and another a multispecific response under specific treatment. Of the patients who only transiently cleared the virus, 1 patient displayed a monospecific response and 1 patient a multispecific response when tested in the first 6 months. Like patient 18, who had a chronic course, the response in patient

12 seemed to increase over time, despite the reappearance of the virus. This observation is also supported by observations in chimpanzee studies, in which CTL responses tended to wane after HCV resolution but gradually expanded in persistent infection [21].

Together our results support recent studies in chimpanzees, which showed that a CTL response broadly directed against multiple epitopes is required for the resolution of aHCV, and indicate that critical factors for resolution are the number of specificities of the $CD8^+$ T cells that are synchronously operational in early infection. This might lower the likelihood of viral escape [21].

Of 19 normal donors, 1 displayed a response against structural and nonstructural HCV epitopes. Another had a positive response directed against 1 E1 epitope. The presence of virus-specific $CD8^+$ T cells in healthy donors, which has been found in a minority of these individuals, suggests either previous exposure to HCV or cross-reactivity to other infectious agents. The detection of virus-specific $CD8^+$ T cells by ELISPOT assay has recently been described by Scognamiglio et al. in HCV-negative healthy family members of patients with chronic HCV infection [22]. Scognamiglio et al. suggested that virus-specific CTL effectors in normal donors may provide protective immunological memory and that the latter requires continuous stimulation by persisting antigen, which either stems from undetectable viral antigen or represents cross-reactivity to self-antigens.

The $CD4^+$ T cell response to NS3 and NS4 protein was measured at identical time points, as was the CD8 T cell response. The fact that a $CD8^+$ T cell response was seen almost exclusively in the face of a strong $CD4^+$ T cell response suggests that a significant $CD4^+$ T cell response is required to mount a detectable $CD8^+$ T cell response. These data are in line with recent observations that $CD4^+$ T cells are needed to enable $CD8^+$ T cells (via antigen-presenting cells) to be activated [23, 24].

In summary, our data indicate that a positive and multispecific $CD8^+$ T cell response during the first 6 months after onset of disease is associated with eradication of HCV infection. Our data also suggest that this response is required to control the disease. A significant and coordinated $CD4^+$ T cell response seems to be a prerequisite for the $CD8^+$ T cell response. The mechanisms of IFN-γ by which this control is mediated (i.e., direct killing of infected cells or interference with viral genome expression, as has been shown in HBV [25]) remains to be clarified. Moreover, the results confirm previous data in HIV and LCMV [4–7] that new technologies, such as the ELISPOT assay, are a powerful tool for use in the analysis and enumeration of virus-specific $CD8^+$ T cells (a tool that does not require long-term in vitro culture). However, in future studies it will be interesting to compare the ELISPOT results with CTL ^{51}Cr-release assays in identical patients. The present data contribute to a better understanding of the virus-host interaction and are a prerequisite for the development of new therapeutic strategies, such as peptide-based or DNA-based vaccines.

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